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Thymineless Death and Ultraviolet Sensitivity in *Micrococcus radiodurans*

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Thymine-requiring mutants of *Micrococcus radiodurans* have been isolated by selection on solid medium containing trimethoprim. Strains requiring either high concentrations of thymine (50 μg/ml) or low concentrations (2 μg/ml) for normal growth were obtained. The Thy⁻ mutant requiring low thymine concentrations has been characterized. It was shown to retain the high ultraviolet light (UV) resistance typical of wild-type *M. radiodurans*, but it was not resistant to thymineless death. Preliminary exposure of the cells to thymineless conditions resulted in enhanced UV sensitivity, and this interaction occurred under conditions where "unbalanced growth" was inhibited by the addition of chloramphenicol. Upon addition of thymine to deprived cells, UV resistance was gradually restored, and this recovery took place in the absence of protein synthesis. A model is proposed to account for the similarity of thymineless death in bacteria whose deoxyribonucleic acid repair efficiencies differ widely.

The extraordinary resistance of *Micrococcus radiodurans* to ionizing and ultraviolet (UV) irradiation is apparently due to its ability to repair damaged deoxyribonucleic acid (DNA). The excision of UV-induced pyrimidine dimers from *M. radiodurans* has been shown to be very efficient (5), and UV-sensitive mutants have been isolated which exhibit reduced rates of cytosine-thymine dimer excision (27). It is therefore inferred that this organism possesses the excision repair mechanism (6, 32, 38) for dealing with damage in its DNA. The repair of X-ray-induced strand breaks in DNA has also been demonstrated in this bacterium (10), and the completion of such repair has been shown to require protein synthesis (11). Setlow and Duggan have shown (37) that 20-fold larger UV doses are required to induce DNA synthesis delays in *M. radiodurans* comparable to those induced in *Escherichia coli* B/r. This may suggest that completion of the repair replication step is also considerably more rapid in this organism than in *E. coli*. Repair replication has, in fact, now been demonstrated in this organism (J. G. Little, *manuscript in preparation*). The ability of *M. radiodurans* to tolerate large doses of UV without loss of viability renders it a useful system in which to study repair replication because the observed modes of DNA synthesis are occurring in cells destined to survive, and therefore it is less likely that aberrant modes of synthesis are present.

The techniques developed in this laboratory to distinguish the repair mode of DNA replication from semiconservative synthesis (17, 32) employ thymine-requiring bacterial mutants to facilitate the incorporation of the density analogue of thymine, 5-bromouracil. No such mutants have been reported for *M. radiodurans*. We report here the isolation and characterization of a thymine-dependent mutant.

Because of the apparent relationship between DNA repair and the phenomenon of thymineless death (31), it was of interest to determine whether this mutant was resistant to thymineless death. Unusual sensitivity to thymineless death has been correlated in *E. coli* in some cases with sensitivity to UV and to deficiencies in DNA repair processes. For example, mutants deficient in polynucleotide ligase are sensitive to thymineless death (30). A mutant deficient in DNA polymerase I is also sensitive to thymineless death in comparison to the parental strain (Bendigkeit and Hanawalt, *unpublished data*). However, mutants deficient in an early step in the excision repair sequence, endonuclease attack, are not unusually sensitive to thymineless death, possibly because single-strand breaks are introduced as a consequence of the thymineless condition itself or because

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naturally occurring breaks persist and accumulate in the absence of thymine. In the present study we demonstrate that *M. radiodurans* is not unusually resistant to thymineless death, and we consider the implications of this fact.

Synergistic interactions of UV and X irradiation have been described in *E. coli* (19) and in *M. radiodurans* (28), indicating that the capacity of cells to repair DNA damage induced by one kind of radiation may be diminished by preliminary exposure to the alternate radiation. Similarly, Gallant and Suskind have shown (15) that thymine starvation of a thymine-requiring *E. coli* mutant enhanced its sensitivity to UV irradiation. We report here that *M. radiodurans* displays a similarly striking loss of UV resistance after thymine starvation and we show that this synergism cannot be ascribed to "unbalanced growth" of the cells in the thymineless condition. Readdition of thymine to thymine-starved cultures permits restoration of UV resistance, and this recovery is shown to occur in the absence of protein synthesis.

MATERIALS AND METHODS

Bacterial strains. Wild-type *M. radiodurans* (2) was used. Mutants requiring high concentrations of thymine (50 μg/ml) for normal growth (MrT_{50}^-) were isolated as described below. Strains exhibiting a low thymine requirement (less than 2 μg of thymine/ml) (MrT_2^-) were derived from these.

Media. A defined medium for cultivating *M. radiodurans* has been described by Raj et al. (34). We used a similar formula containing, in addition, folic acid and vitamin B_{12}. This minimal medium contained (per liter): KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 10 mg; $MnSO_4 \cdot H_2O$, 7.6 mg; $(NH_4)_2HPO_4$, 0.5 g; glucose, 5.0 g; L-glutamic acid, 0.5 g; L-methionine, 10 mg; thiamine hydrochloride, 10 μg; biotin, 10 μg; niacin, 250 μg; pyridoxine, 200 μg; vitamin B_{12}, 200 μg; folic acid, 10 μg. Bacterial growth in this medium was stimulated by the addition of 0.1% vitamin-free Casamino Acids (see Results). In the experiments described here, except where indicated, this supplemented minimal medium (SM medium) was used.

SM agar plates were prepared by solidifying SM medium with 10 g of agar per liter. Plates used in mutant selection contained, in addition, thymine (50 μg/ml) and trimethoprim (100 μg/ml). Plates containing thymine at a concentration of 50 μg/ml or 5 μg/ml were used for cultivating mutants with high and low thymine requirements, respectively. TGY agar contained (in grams per liter): tryptone, 5; glucose, 1; yeast extract, 3; agar, 10. For TGY top agar, the agar concentration was reduced to 7 g/liter.

Measurement of growth. Except where indicated, cells were grown at 37 C in a shaker bath in 20-ml cultures in balanced growth at concentrations to about 2×10^7 colony-forming units per ml. Optical densities of cultures were measured in a Zeiss PMQ II spectrophotometer at 650 nm in 1-cm path-length cells. An optical density of 0.2 corresponded to a viable cell count of approximately 1.5×10^7 colony-forming units/ml. Viable cell count was determined by the pour plate method. Samples were diluted appropriately in 0.1 M phosphate buffer (*p*H 7.0), and 0.1-ml samples were added to TGY plates, which were immediately overlaid with 3.0 ml of TGY top agar maintained at 44 C. Colonies were counted after at least 3 days of incubation at 37 C. Viable count estimates for *M. radiodurans* have usually been performed by the spreading procedure. Our comparison of these two methods gave equivalent estimates of viable colonies.

Methods of mutant isolation. The method devised by Stacey and Simson (40) for isolating thymine-requiring mutants of *E. coli* was used. Cells were grown in SM medium containing 20 μg of trimethoprim per ml. Thymine (50 μg/ml) was added, and the culture was grown for 5 days, by which time cell concentrations in excess of 10^8 bacteria per ml were obtained. Clones derived from this culture were tested for thymine dependency by replica plating. This selection procedure was also adapted to solid medium in the following manner. A culture of wild-type *M. radiodurans* was grown to stationary phase in SM medium lacking thymine. Samples of the culture containing $\sim 5 \times 10^7$ colony-forming units were spread on plates to which thymine and trimethoprim had been added. After 2 days of growth at 37 C, colonies of resistant bacteria (usually 50 to 100) were visible against a faint background growth. Colonies were picked, purified by streaking on thymine-containing agar, and then tested for thymine dependency. By this method, mutants with a high (50 μg/ml) thymine requirement were obtained. Strains capable of growth in low concentrations of thymine were isolated from these auxotrophs either by heavy seeding on plates containing 5 μg of thymine per ml or by the method described by Harrison (18). We thank G. H. Hitchings of Burroughs Wellcome and Co. for a gift of trimethoprim.

Thymine starvation. A mutant of *M. radiodurans* having a low thymine requirement (MrT_2^-) was grown in SM medium supplemented with thymine at 2 μg/ml. Exponentially growing cells were centrifuged and washed three times with 0.1 M phosphate buffer. The cells were then suspended in 40 ml of SM medium at 37 C lacking thymine. To 20 ml of this culture, thymine was added back at a concentration of 2 μg/ml. At various times, samples were removed for turbidity measurements and viable count estimation.

UV irradiation. Cells grown to exponential phase in SM medium containing thymine (2 μg/ml) were harvested, washed, and resuspended in 20 ml of phosphate buffer. Irradiation was performed with a Westinghouse 15-w germicidal UV lamp as previously described (21). The intensity was 19.5 ergs per mm^2 per sec. Samples were removed at various times, diluted appropriately in chilled TGY, and plated on TGY agar. Colonies were counted after 3 days of incubation at 37 C.

RESULTS

Isolation of thymine-requiring mutants of M. radiodurans. After 5 days of growth of wild-type *M. radiodurans* in liquid selection medium containing 20 μg of trimethoprim per ml, less than 2% of the colony formers tested exhibited thymine dependency. Moreover, the stability of the isolates obtained was generally unsatisfactory. Prior mutagenic treatment of the wild-type cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg et al. (1) resulted in improved yields of mutants, but the stability of the isolates remained unsatisfactory.

After testing growth inhibition by trimethoprim at various concentrations, we found that by incorporating it into solid medium at 100 μg/ml (as described in Materials and Methods) a simple and effective selection was obtained. The large yield of independent mutants afforded by this method greatly facilitates the selection of stable auxotrophs. When tryptone (0.1%) was used instead of vitamin-free Casamino Acids, growth of the background lawn was more luxuriant, and outgrowth of colonies was much smaller. With unsupplemented minimal plates, no growth was detected under these conditions. From these auxotrophs, strains requiring low thymine concentrations were isolated as described above. When plates containing low thymine were seeded with $\sim 4 \times 10^8$ colony-forming units, about 300 to 400 colonies were observed after 3 days of incubation at 37 C. It is known (29) that in *E. coli* the secondary mutation to low thymine requirement is a single-step mutation. An estimate of the proportion of mutant colonies detected in this study ($\sim 1 \times 10^{-6}$) suggests that this is also the case with *M. radiodurans*.

Characterization of mutant MrT_2^-. The auxotroph selected for further study exhibited an absolute requirement for thymine. No revertants were obtained either by prolonged growth in liquid medium lacking thymine or by inoculating thymineless plates with $\sim 10^9$ cells.

Absorbancy measurements of growth in the presence and absence of thymine are shown in Fig. 1. In the absence of thymine, an initial increase in absorbancy was followed by a steady decline. Samples from the thymine-starved culture were observed by phase-contrast microscopy. During the first 4 hr of starvation, the cells became enlarged while maintaining their characteristic tetrad appearance. Thereafter, an increasing number of gross spheroplast forms were visible, presumably reflecting a loss of cell wall integrity. Readdition of thymine at 10 μg/ml after prolonged starvation gave rise to accelerated lysis (Fig. 1). The magnitude of this latter effect varied considerably in our experiments.

Thymineless death. Figure 2 shows the

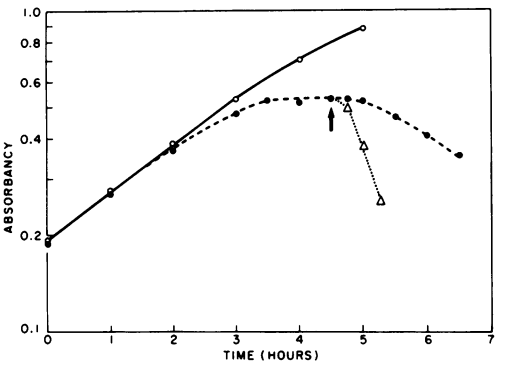

FIG. 1. *Growth of M. radiodurans, strain MrT_2^-, in the presence (○) and absence (●) of thymine. After balanced growth in supplemented minimal medium (SM) containing thymine (2 μg/ml), cells were washed and transferred to 40 ml of SM medium lacking thymine. To 20 ml of this culture, thymine was added at 2 μg/ml, and both cultures were incubated at 37 C. At the time indicated, thymine (10 μg/ml) was added to a portion of the starved culture, and absorbancy change was measured (△).*

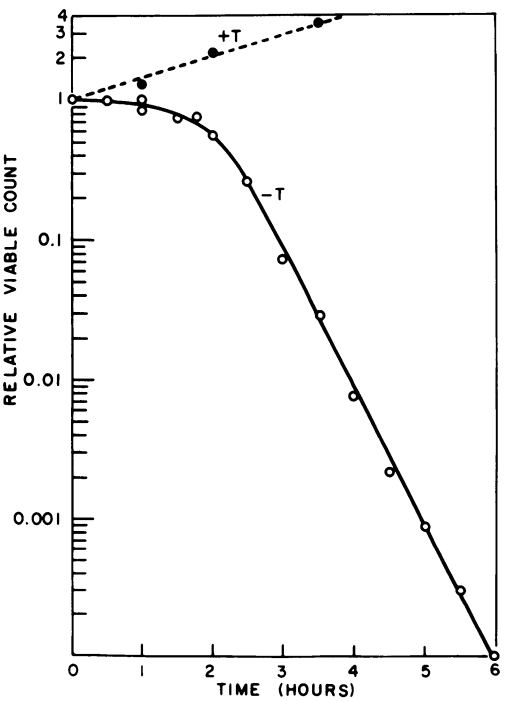

FIG. 2. *Viability of strain MrT_2^- in the presence and absence of thymine. Conditions as in Fig. 1.*

survival of the thymine-requiring mutant in the presence and absence of thymine. After an initial lag period, loss of viability continued exponentially for at least four decades.

UV resistance. To ascertain that the thymine-dependent strain retained the high UV resistance characteristic of the parental strain, UV survival curves were determined (Fig. 3). When cultivated in TGY medium and irradiated, the cells exhibited the high resistance typical of the wild-type strain (37). However, when grown in SM medium supplemented with thymine (2 μg/ml), the UV-resistance of MrT_2^- was markedly reduced. Krabbenhoft et al. have reported (23) that, when wild-type *M. radiodurans* was grown in TGY to which NZ-Case (a tryptic digest of casein) had been added, a 10-fold increase in UV sensitivity occurred.

The presence of Casamino Acids in our medium may be responsible for the enhancement in UV sensitivity that we observed.

Effect of thymine starvation upon UV resistance. When cells were incubated for varying periods of time in SM medium lacking thymine before irradiation, a progressive loss of UV resistance was observed (Fig. 4). The most striking aspect of these data was that the effect of thymine starvation was a progressive erosion of the initial shoulder while the exponential portion of the survival curve was unaffected. A similar pattern has been described by Moseley and Laser (28) in their study of UV, X-ray synergistic interactions in *M. radiodurans*. It is worth noting that this increase in UV sensitivity occurs within a period of 60 min of thymine deprivation, which by itself does not result in cell death.

Relationship between unbalanced growth and UV sensitivity. Thymine deprivation in thymine-dependent cells gives rise to unbalanced growth (8). To determine whether or not protein synthesis or cell enlargement or both are implicated in the resulting sensitization to UV, cells transferred to thymineless medium were divided into two portions and incubated in the presence or absence of chloramphenicol (15 μg/ml) for 60 min. Boling and Setlow have reported that chloramphenicol at a concentration of 5 μg/ml inhibits amino acid incorporation in *M. radiodurans* (5). In the presence of chloramphenicol, no change in the absorbancy of the culture occurred during thymineless incubation. Furthermore, the presence of chloramphenicol for this period did not affect the viable cell count.

As shown in Fig. 5, the effect of thymine deprivation on the enhancement of UV sensitivity is the same in the presence or absence of protein synthesis. Thus, the effect is not explained by any model involving gross change in cell size or protein content.

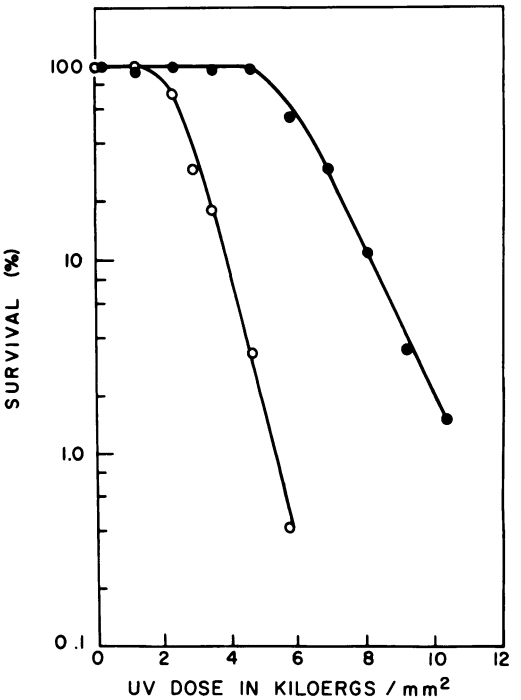

FIG. 3. *Survival curves of strain MrT_2^- exposed to UV irradiation at 254 nm. Cells were grown in TGY broth (●) or in SM medium containing thymine (○). Washed cells were irradiated in 0.1 M phosphate buffer at room temperature, and postirradiation incubation conditions were identical for both cultures (see Materials and Methods).*

Recovery from thymineless sensitization. After thymine deprivation for a period of 60 min, a culture of strain MrT_2^- was divided into three portions. A UV survival curve was determined for one portion immediately. Thymine at 2 μg/ml was added to each of the other portions, and to one of these chloramphenicol (15 μg/ml) was also added. These cultures were then incubated for 60 min before irradiation. As shown (Fig. 6) recovery of UV resistance occurred in both cultures, and the effect was in fact somewhat more pronounced in the culture in which protein synthesis had been inhibited.

DISCUSSION

The incorporation of trimethoprim into supplemented plates containing thymine provided a convenient selection medium for the isolation of thymine-requiring mutants of *M. radiodurans*. The yield of independent mutants obtained by selection on solid media greatly

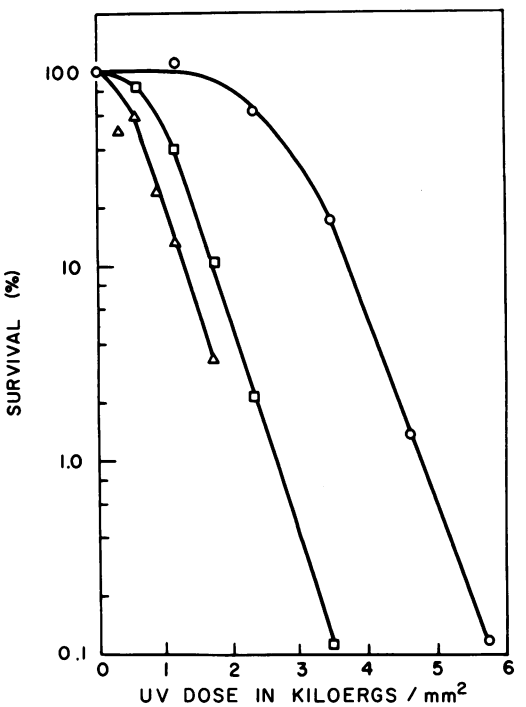

FIG. 4. *UV inactivation curves of MrT_2^- cells after growth in thymineless medium. After growth in SM medium containing thymine, washed log-phase bacteria were either irradiated immediately* (○), *or grown in SM medium lacking thymine for periods of 60 min* (□) *or 120 min* (△) *before irradiation.*

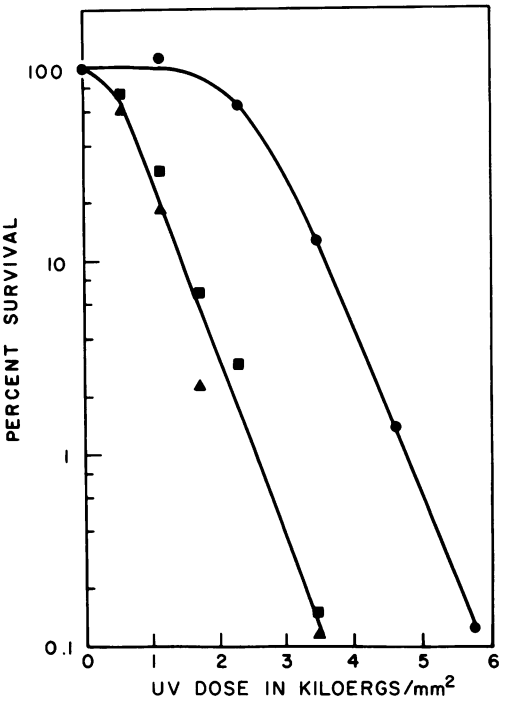

FIG. 5. *Changes in UV sensitivity of strain MrT_2^- bacteria after 60 min of growth in thymineless medium in the presence* (■) *or absence* (▲) *of chloramphenicol (15 μg/ml). A UV survival curve of an unstarved culture (see Fig. 4) is included for comparison* (●).

increases the chances of isolating suitable mutants. Caster has described a method (7) for isolating Thy⁻ *E. coli* strains which is similar in principle to that used here.

We have shown that the behavior of *M. radiodurans* Thy⁻ under conditions of thymine deprivation is similar to that of thymine-dependent strains of *E. coli* (8, 24) and mycoplasma (39). A two- to threefold increase in cell mass occurs under thymineless conditions, and this is followed by a plateau. However, with *M. radiodurans*, the eventual appearance of enlarged spheroplasts and the steady decline in turbidity with prolonged starvation suggest that the cell wall of the micrococcus may be more easily disrupted by unbalanced growth. The accelerated lysis observed upon readdition of thymine to starved cultures is reminiscent of the thymineless induction of prophage in *E. coli* K-12(λ) (22). No phages of *M. radiodurans* have been reported. Moreover, since most laboratory stocks of the bacterium have been derived from a common source (2) it would be difficult to detect lysogeny in the absence of indicator strains. Filtrates of lysed cultures from these experiments were tested with various strains of *M. radiodurans*. No plaques were observed. At this time it seems more probable that readdition of thymine and, presumably, resumption of DNA synthesis at a time when the cell wall is already damaged may result in more rapid cellular disruption. However, we can offer no explanation for the association of cell wall damage with thymine starvation.

The survival curve shown in Fig. 2 demonstrates that *M. radiodurans* is not unusually resistant to thymineless death. When allowance is made for the long generation time of *M. radiodurans* (120 min), compared to 35 min for *E. coli*, the kinetics of thymineless death appear quite similar (24). Thus in *E. coli* the 30-min "lag" period corresponds to 0.86 generation, and in *M. radiodurans* the 2-hr "lag" period corresponds to 1 generation. The half-life for killing in the exponential portion of the survival curve is about 10 min (or 0.33 generation) for *E. coli* and about 20 min (or 0.17 generation) for *M. radiodurans*.

A number of hypotheses have been advanced in an effort to explain the mechanism of thy-

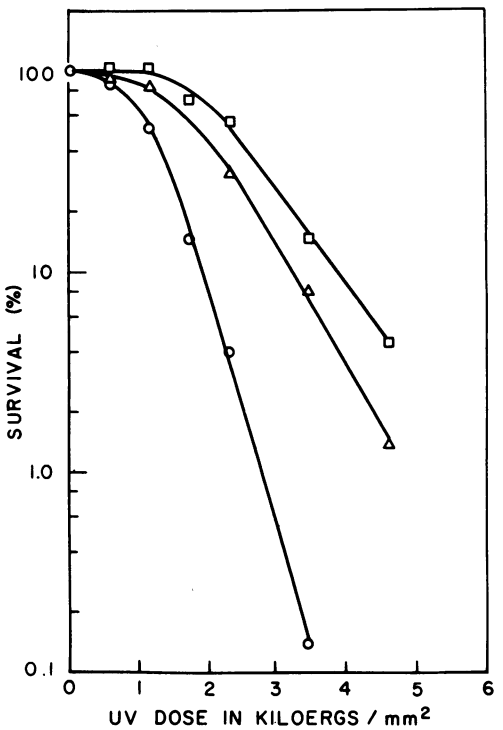

FIG. 6. *Recovery of UV resistance of strain MrT_2^- upon restoration of thymine to starved cells. After thymine deprivation for a period of 60 min, the cells were either irradiated immediately* (○) *or grown for a further 60 min in the presence of thymine at 2 μg/ml, with* (□) *or without* (△) *chloramphenicol.*

mineless death in bacteria. These include proposals that cell death is a consequence of unbalanced growth (4, 8), episome induction (20, 25), and DNA damage (26, 31). Since thymine deprivation is also known to be mutagenic (9) and recombinogenic (14), the hypotheses invoking DNA damage as the most crucial consequence of thymine starvation have been attractive and have stimulated studies of the nature of such damage. DNA strand breaks have been reported to accumulate in *E. coli* during thymine deprivation (13, 41). However, a contrary finding has been reported (3) for *E. coli* C Thy^-, in which no DNA strand breaks were detected during thymineless incubation. In *Bacillus subtilis* Thy^-, decay of nascent DNA followed by more general DNA degradation has been observed during thymine starvation (35). Preferential degradation of nascent DNA during thymine starvation has also been observed in *E. coli* by Nunberg and Hanawalt (*unpublished data*).

While it is obviously important that the nature of DNA damage induced by thymine deprivation should be elucidated, it remains to be established that the reported damage is biologically significant, i.e., a primary cause of cell death. It is of interest that, upon readdition of thymine to cells after a period of thymineless growth, DNA synthesis resumes immediately and at a higher initial rate than before starvation (12, 33). If DNA damage accumulates during thymine deprivation, it seems that these lesions do not constitute a significant block to DNA synthesis, unlike those induced by radiation and some mutagenic treatments. The increased rate of DNA synthesis observed after thymine deprivation is at least in part due to reinitiation of new cycles of DNA replication (33). It has been suggested that DNA repair may be inhibited during thymine starvation, either by the absence of required thymine for repair replication (31), or by inhibition of polynucleotide ligase (13), possibly by adenosine triphosphate (ATP) accumulation in the cells. Upon restoration of thymine (e.g., by plating the bacteria), the capacity to repair DNA damage would then be restored.

The fact that thymine restoration results in the immediate resumption of DNA synthesis suggests an explanation for thymineless death that has not been explicitly considered; that upon restoration of thymine, the normal growing point region of DNA may encounter damaged regions of the DNA before they are repaired, with possibly lethal results. A similar model has been proposed by Hanawalt (16) to explain the enhanced resistance to UV of *E. coli* cells that have completed the normal DNA replication cycle in the absence of protein synthesis. The emphasis of this view is that the DNA damage only becomes significant (i.e., lethal) because of the combination of two sequential conditions: (i) the inhibition of DNA repair during thymine deprivation, and (ii) the brevity of time available for repair when thymine is restored.

The results obtained in the present study are consistent with this model. *M. radiodurans*, which is capable of repairing large numbers of UV- and X-ray-induced DNA lesions, is not resistant to thymineless death. This becomes explicable if the time available for repair is short, i.e., the time between the resumption of DNA synthesis—and repair capacity—and the time at which the growing point encounters a potentially lethal damaged region. Because of the limited time available for repair, the possession of unusual repair *capacity* may not be especially advantageous under these circumstances, since it cannot be adequately expressed. On the other hand, certain kinds of

repair deficiency may confer sensitivity to thymineless death, e.g., those found in DNA polymerase I and ligase-deficient strains, since the limited repair time available cannot be effectively utilized in these cases.

Thymine starvation renders *M. radiodurans* Thy^- sensitive to UV irradiation. The finding that this sensitization also occurs in the absence of protein synthesis indicates that overall cell growth and unbalanced protein synthesis is not of primary importance in this interaction. The shape of the survival curves obtained by UV irradiation of thymine-starved cultures is consistent with an effect on DNA repair capacity (19), i.e., the loss of the shoulder without a concomitant change in exponential slope. In principle this could reflect either a *saturation* of repair capacity by the additive effects of DNA lesions due to thymine starvation and those induced by UV, or an *inhibition* of repair resulting from the thymineless episode.

The recovery of UV resistance after thymine restoration (Fig. 6) is shown to occur even when protein synthesis is blocked. This indicates that the sensitization effected by thymine deprivation is not due to a loss of repair capacity (e.g., enzyme loss), but rather to its inhibition. The fact that the chloramphenicol-treated culture appears more resistant than the untreated culture could be attributed to the increased UV resistance of cells which have completed replication cycles.

We cannot clearly rule out either of the two models suggested for the synergistic effect of thymine deprivation upon UV sensitivity. Although the accumulation of repairable lesions in the absence of thymine is a possible explanation, we prefer the second model, that somehow the excision repair mechanism is inhibited indirectly (e.g., by ATP accumulation) in the absence of thymine. Continuing studies are designed to test this possibility.

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